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Published in:
Biochemistry

DOI:
10.1021/bi953005v

Publication date:
1996

Purification and Reconstitution of the Glutamate Carrier GltT of the Thermophilic Bacterium *Bacillus stearothermophilus*†

Isabelle Gaillard,‡ Dirk-Jan Slotboom,‡ Jan Knol, Juke S. Lolkema,* and Wil N. Konings

Department of Microbiology, Groningen Biotechnology and Biomolecular Sciences Institute, University of Groningen, The Netherlands

Received December 19, 1995; Revised Manuscript Received February 28, 1996⊗

ABSTRACT: An affinity tag consisting of six adjacent histidine residues followed by an enterokinase cleavage site was genetically engineered at the N-terminus of the glutamate transport protein GltT of the thermophilic bacterium *Bacillus stearothermophilus.* The fusion protein was expressed in *Escherichia coli* and shown to transport glutamate. The highest levels of expression were observed in *E. coli* strain DH5α grown on rich medium. The protein could be purified in a single step by Ni^{2+}-NTA affinity chromatography after solubilization of the cytoplasmic membranes with the detergent Triton X100. Purified GltT was reconstituted in an active state in liposomes prepared from *E. coli* phospholipids. The protein was reconstituted in detergent-treated preformed liposomes, followed by removal of the detergent with polystyrene beads. Active reconstitution was realized with a wide range of Triton X100 concentrations. Neither the presence of glycerol, phospholipids, nor substrates of the transporter was necessary during the purification and reconstitution procedure to keep the enzyme in an active state. In *B. stearothermophilus*, GltT translocates glutamate in symport with protons or sodium ions. In membrane vesicles derived from *E. coli* cells expressing GltT, the Na^+ ion dependency seems to be lost [Tolner, B., Ubbink-Kok, T., Poolman, B., & Konings, W. N. (1995) *Mol. Microbiol. 18,* 123−133], suggesting a role for the lipid environment in the cation specificity. In agreement with the last observation, glutamate transport catalyzed by purified GltT reconstituted in *E. coli* phospholipid is driven by an electrochemical gradient of H^+ but not of Na^+.

Bacillus stearothermophilus is a thermophilic bacterium that grows optimally at 63 °C. Proteins of thermophiles are likely to be more stable than those of their mesophilic counterparts. This may be of particular importance in the study of membrane proteins when they are taken out of their natural phospholipid environment. Glutamate uptake in *B. stearothermophilus* is mediated via a secondary transporter termed GltT. Translocation of glutamate is coupled to translocation of two cations, a proton and a sodium ion, and, therefore, is driven by the proton and sodium ion motive forces (Heyne et al., 1991). The gene coding for the transporter GltT has been cloned and sequenced (Tolner et al., 1992a), and the deduced amino acid sequence showed significant homology with the members of a large family of carboxylic acid transporters in which carriers from related organisms like the glutamate transporter GltP from *Escherichia coli* are found (Tolner et al., 1992b) but also carriers from eukaryotic origin like the glutamate transporter from mammalian brain [e.g. Pines et al. (1992)]. The transporters in this family differ both in substrate specificity and cation specificity.

The *gltT* gene was expressed in a strain of *E. coli* deficient in glutamate transport. Studies with membrane vesicles derived from the recombinant strain revealed a surprising result. Expression of GltT in *E. coli* resulted in complete loss of the sodium ion dependency; glutamate transport was driven only by the proton motive force. Similar studies with membrane vesicles from *B. stearothermophilus* showed an increase in uptake activity in the presence of a sodium ion motive force, a feature that was most apparent at elevated temperatures (Tolner et al., 1995). The data suggest that, in addition to the primary sequence, the cation specificity of a carrier may depend also on conformation. Subtle conformational differences of GltT induced by the lipid environment and/or temperature may select for proton or sodium ion as the symported cation. Implicitly, it would mean that the difference between a proton and a sodium ion binding site on the carrier is extremely small.

The importance of these findings requires that they are confirmed with the purified protein reconstituted in proteoliposomes. The isolated protein can be reconstituted in phospholipids from different origins and studied with respect to their cation selectivity at different temperatures. In addition, purification of the protein is essential to determine conformational changes by spectroscopic techniques. Here, we report on the first step in these studies, the purification and functional reconstitution of GltT in *E. coli* phospholipids. After optimization of the expression of the *gltT* gene in *E. coli*, the protein is purified in a single step by His tag affinity chromatography (Hochuli et al., 1987) and reconstituted by the detergent titration technique (Rigaud & Pitard, 1995). Specific precautions that have been reported for the suc-

† This work was supported in part by a grant from the HCM program of the EEC (to I.G.) and by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, and the Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research program of the Netherlands Association of Biotechnology Centres in the Netherlands (ABON) (to D.-J. S.).

* Corresponding author: Department of Microbiology, University of Groningen, Kerklaan 30, 9751NN Haren, The Netherlands. Phone: (31)503632155. Fax: (31)503632154. E-mail: j.s.lolkema@biol.rug.nl.

‡ The first two authors have equally contributed to this paper.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0006-2960(95)03005-4 CCC: $12.00

Table 1: DNA Sequences of the Oligonucleotides Used in This Study[a]

purpose	DNA sequence
construction pKS*nco*I	5′-AATCATGG**C**CAT**G**GCTGTTTC-3′ [$]
His tag linker (sense)	5′-CATG**CATCACCATCACCATCAC**GATGACGATGACAAAGCCATGGGG-3′ [#]
His tag linker (antisense)	5′-GATCCCCCATGGCTTTGTCATCGTCATC**GTGATGGTGATGGTGATG**-3′ [#]
PCR gltT (forward)	5′-GAAAGGGGCGAAT**CCATGG**GAAAAATTGGATTA-3′ [%]
PCR gltT (backward)	5′-AAGCTGTCCTTTT**TCTAGA**AGGACAGCT-3′ *

[a] Indicated in bold are the base ([$]) changes, the six histidine codons ([#]) the *Nco*I ([%]) and *Xba*I (*) restriction sites.

cessful purification and reconstitution of several transporters from mesophiles were not necessary in the case of GltT (see Discussion). This may reflect the higher thermostability of this membrane protein from a thermophilic organism.

MATERIALS AND METHODS

Materials

Ni^{2+}-NTA resin was obtained from QIAGEN and L-[^{14}C]-glutamate from Amersham, U.K.; synthetic oligonucleotides were obtained from Eurosequence, Groningen, The Netherlands. All other materials were obtained from commercial sources.

Bacterial Strains and Growth Conditions

The following *E. coli* strains were used: DH5α, JM101, and ECOMUT1. The latter strain lacks the genes coding for the glutamate transporters GltP and GltS (Tolner et al., 1995). Unless stated otherwise, the cells were grown at 37 °C in LB medium or minimal medium supplemented with 20 mM glycerol and 0.2% casamino acids. The antibiotics ampicillin and kanamycin were included when appropriate at concentrations of 100 and 50 μM, respectively.

Construction of the Expression Vector pGltThis

Construction of the Host Vector pKShis. All genetic engineering was done using the standard procedures described in Sambrook et al. (1989). A *Nco*I restriction site (CCATGG) was introduced around the start codon of the *lacZ* gene on phagemid pBlueScript II KS (Stratagene, La Jolla, CA) by site-directed mutagenesis using the Kunkel method, yielding vector pKS*nco*I. The base sequence of the mutagenic primer and the other oligonucleotides used in this study are listed in Table 1. Transformants were screened by restriction analysis of the plasmids. Subsequently, the unique *Cla*I site in the multiple-cloning site of pKS*nco*I was opened, and the ends were made blunt by filling in the overhangs with Klenow polymerase. Ligation yields vector pKS*nco*IΔClaI. Deletion of the *Cla*I site results in a frame shift, making the downstream *lacZ* sequences out of frame and resulting in white colonies on XGal plates. The frame shift was restored by digesting pKS*nco*IΔclaI with *Nco*I and *Bam*HI, which removes the destroyed *Cla*I region, and inserting a DNA linker coding for a His tag of the appropriate length. Successful insertion of the linker was concluded from blue coloring on XGal plates. The 5′ end of the linker has an overhang compatible with a *Nco*I cleavage site but destroys the site after ligation, while the 3′ end of the linker is compatible with a *Bam*HI cleavage site. The linker codes for a sequence of six histidines followed by an enterokinase proteolytic cleavage site. The coding regions are in frame with the *lacZ* gene. In front of the *Bam*HI site, the linker contains a *Nco*I site (CCATGG) with the ATG bases in the same reading frame as the *lacZ* start codon. The linker was made by mixing 5 μg of two complementary synthetic oligonucleotides for 5 min at 37 °C (Table 1). The resulting vector is termed pKShis.

Table 2: Primary Sequences of the N-Terminal Part of the GltT Constructs

protein	amino acid sequence[a]
GltT	MRKIG...
GltT*nco*I	**M**G**KIG...**
GltThis	MHHHHHHDDDDKA**M**G**KIG...**

[a] The original GltT residues are indicated in bold, and the enterokinase site is underlined.

Cloning of gltT into pKShis. Plasmid pGBT102 containing the *gltT* gene has been described by Tolner et al. (1992a) and was used to amplify the gene by the polymerase chain reaction (PCR) technique. Two primers were used to amplify the 1.29 kb DNA fragment coding for GltT and to insert an *Nco*I cleavage site at the start codon of the gene and an *Xba*I site 25 nucleotides downstream of the stop codon (Table 1). The amplification results in a mutation in the second codon of *gltT* (Table 2). The purified PCR fragment was restricted with the *Nco*I and *Xba*I restriction enzymes and ligated into pKShis and pKS*nco*I digested with the same enzymes. The resultant vectors are termed pGltThis and pGltT*nco*I, respectively. Positive transformants were selected by blue/white screening and restriction analysis of the plasmids. The inserts were sequenced using an Amersham automated sequencer.

Measurement of Expression Levels

Cells from 10 mL cultures were harvested in the late log phase, washed once with 50 mM potassium phosphate (pH 8), and, subsequently, resuspended in the same buffer containing 600 mM NaCl. The suspension was supplemented with 1% Triton X100 and left at room temperature for 30 min. Undissolved material was removed by ultracentrifugation for 10 min at 100000*g*. The supernatant was mixed with 100 μL of Ni^{2+}-NTA resin and incubated for 30 min under continuous shaking. The resin was recovered by a short spin and washed once with 1 mL of buffer and eluted with 0.2 mL of buffer containing 250 mM imidazole. The eluate was loaded onto a 10% sodium dodecyl sulfate (SDS)−polyacrylamide gel that was stained with silver.

Purification of GltThis

Cells from a 6 L culture in LB medium harvested at a OD_{660} of about 0.7 were washed once with 250 mL of 50 mM potassium phosphate (pH 8) and resuspended in 40 mL of the same buffer containing 1 mM $MgSO_4$ and a trace amount of deoxyribonuclease. Cells were broken by three passages through a French Press cell operated at 10 000 psi

at 4 °C. Unbroken cells and debris were removed by centrifugation at 10000*g* for 10 min at 4 °C, and the membranes were collected from the supernatant by centrifugation at 100000*g* for 90 min at 4 °C. Membranes were washed once with 50 mL of potassium phosphate (pH 8) and 1 M NaCl and resuspended in 50 mM potassium phosphate (pH 8) at a protein concentration of 10 mg/mL (Lowry et al., 1952). The membrane suspension was stored in liquid nitrogen. Membranes (4 mg/mL) were solubilized in 50 mM potassium phosphate (pH 8), 400 mM NaCl, 20% glycerol, and 1% Triton X100. The solution was left on ice for 1 h with intermittent agitation. Undissolved material was removed by centrifugation at 250000*g* for 20 min at 4 °C. The supernatant was mixed with Ni^{2+}-NTA resin (100 μL per 10 mg of protein) equilibrated in 50 mM potassium phosphate (pH 8), 300 mM NaCl, 10% glycerol, 0.1% Triton X100, and 10 mM imidazole and incubated for 1 h at 4 °C under continuous shaking and, subsequently, poured into a column. The column was washed with 1 mL of equilibration buffer containing 40 mM imidazole. The protein was eluted with 0.5 mL of the same buffer containing 150 mM imidazole and immediately used for reconstitution.

Reconstitution of GltThis in Proteoliposomes

Reconstitution was performed essentially as described by Knol et al. (1996). A 3:1 (w:w) mixture of *E. coli* total lipid extract and egg yolk phosphatidylcholine was resuspended in 50 mM potassium phosphate (pH 7) at a final concentration of 20 mg of lipid per milliliter. The suspension was frozen in liquid nitrogen, thawed slowly, and extruded through 400 nm polycarbonate membranes (Avestin). The liposomes thus obtained were diluted 6-fold in the same buffer and treated with increasing amounts of Triton X100. The solubilization of the liposomes was followed by measuring the optical density at 540 nm (Rigaud & Pitard, 1995). Liposomes treated with different amounts of the detergent were mixed with purified GltT at a ratio of 10 μg of protein per 5 mg of lipid. The mixture was left at room temperature for 30 min, after which the detergent was removed by three successive extractions with polystyrene beads (BioBeads, 80 mg/mL). The first extraction was done at room temperature for 2 h, and the second and third were done at 4 °C for 2 and 16 h, respectively. The beads were removed by filtration over glass wool, and the proteoliposomes were recovered by centrifugation at 250000*g* for 20 min. The proteoliposomes were resuspended in the appropriate buffer and stored in liquid nitrogen.

Transport Assays

Right-Side-Out (RSO) Membrane Vesicles. Membrane vesicles of *E. coli* strains DH5α and ECOMUT1 were prepared by the osmotic lysis procedure as described by Kaback (1971). The membranes were resuspended in 50 mM potassium phosphate (pH 6) at a protein concentration of 15 mg/mL and stored in aliquots in liquid nitrogen. The membrane vesicles were energized by the potassium ascorbate/phenazine methosulfate (PMS) electron donor system. The membranes were diluted to a concentration of 0.6 mg/mL in 50 mM potassium phosphate (pH 6), 2 mM $MgSO_4$, and 10 mM potassium ascorbate. The experiments were performed in 100 μL at 30 °C under a constant flow of water-saturated air in samples of 100 μL. PMS was added at a concentration of 100 μM, and the proton motive force was allowed to develop for 1 min, after which L-[^{14}C]glutamate was added to a final concentration of 1.9 μM. The uptake was stopped by adding a 20-fold volume of ice cold 0.1 M LiCl solution, followed by immediate filtration over cellulose nitrate filters (0.45 μm pore size). The filters were washed once with 2 mL of 0.1 M LiCl and assayed for radioactivity.

Proteoliposomes. For counterflow experiments, proteoliposomes were loaded with 50 mM potassium phosphate (pH 7), 5 mM potassium glutamate, and 2 mM $MgSO_4$ by freezing, thawing, and extrusion through polycarbonate filters (400 nm pore size). The proteoliposomes were collected by centrifugation and resuspended in the same buffer at a concentration of approximately 100 mg of lipid per milliliter. Counterflow was initiated by diluting the proteoliposomes 75-fold in the same buffer without unlabeled glutamate but with 1.3 μM L-[^{14}C]glutamate. The uptake was stopped as described above.

For assays of L-glutamate uptake driven by artificial gradients, the proteoliposomes were washed twice with 20 mM morpholineethanesulfonic acid (Mes) (pH 6) and 100 mM potassium acetate and concentrated as described above. Proton motive force-driven uptake was initiated by diluting the proteoliposomes 75-fold in 120 mM Mes, 100 mM methylglucamine, 0.7 μM valinomycin, and 1.3 μM L-[^{14}C]-glutamate prewarmed at 30 °C. Both a proton motive force and a sodium ion motive force were created by dilution into the same buffer containing 100 mM NaOH instead of methylglucamine. Control experiments were performed by diluting the proteoliposomes into the buffer with which they were loaded.

RESULTS

Construction of the Expression Vector pGltThis. A derivative of pBlueScript pKS II was constructed that facilitates the construction of genes fused downstream of a sequence coding for six histidines (His tag) and an enterokinase cleavage site. The vector, pKShis, was constructed in two steps as described in detail in Materials and Methods. In pKShis, downstream of the *lac* promoter are the ribosomal binding site and the start codon of the *lacZ* gene followed by in frame sequences coding for the His tag and the enterokinase cleavage site and a *Nco*I restriction site (CCATGG) with the ATG bases in frame with the *lacZ* start codon. Downstream of the *Nco*I site are the *Bam*HI to *Sac*I restriction sites of the original pBlueScript multiple-cloning site. The downstream *lacZ*α sequences are in frame with the *lacZ* start codon and allow blue/white screening of inserts on XGal indicator plates.

The 1.3 kb gene coding for the glutamate carrier of *B. stearothermophilus*, GltT, was amplified by the PCR technique from plasmid pGBT102 (Tolner et al., 1992a) using a forward primer that introduces an *Nco*I cleavage site around the start codon and a backward primer that introduces an *Xba*I site downstream of the stop codon. The *Nco*I site results in the Arg2Gly mutation. The PCR product was cloned into pKShis, yielding pGltThis. Under control of the lac promoter, pGltThis codes for GltT with a N-terminal His tag and an enterokinase cleavage site. In addition, the PCR product was cloned into vector pKS*nco*I which results in the same construct except that the sequences coding for the His tag and the enterokinase site are missing (construct

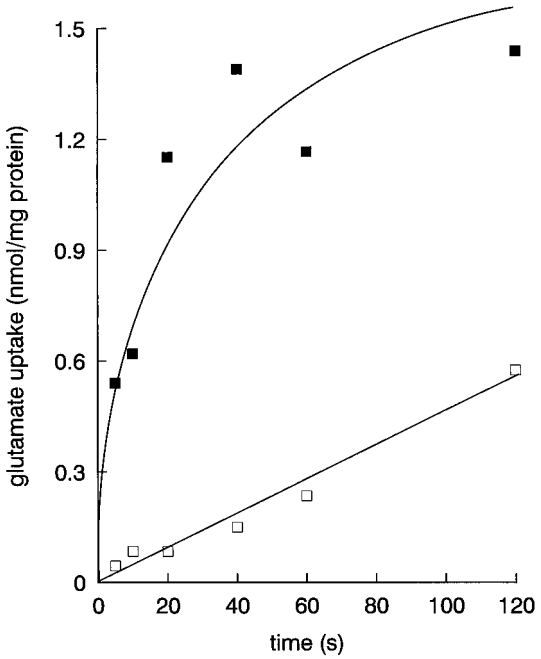

FIGURE 1: Glutamate uptake catalyzed by GltThis. Right-side-out membrane vesicles were prepared from *E. coli* DH5α cells harboring plasmids pGltThis (■) and pKShis (□) and assayed for glutamate uptake as described in Materials and Methods.

pGltT*nco*I). The N-terminal amino acid sequences of the different constructs are indicated in Table 2. The inserts in the two plasmids were sequenced and found to be the same as the published base sequence of the *gltT* gene (Tolner et al., 1992a) except for the changes indicated in Table 2.

Activity of GltThis. Plasmid pGltThis and pGltT*nco*I, coding for the GltT transporter with and without the His tag, respectively, were transformed to *E. coli* DH5α. Membrane vesicles of the recombinant strains were prepared and assayed for glutamate uptake activity. Since *E. coli* DH5α expresses its own glutamate carriers (Deguchi et al., 1989; Tolner et al., 1992b), membrane vesicles from DH5α transformed with plasmid pKShis were used as a control. Figure 1 shows that the initial rate of proton motive force-driven uptake of glutamate in membrane vesicles derived from DH5α expressing GltThis is significantly higher than in the control vesicles, indicating that the His-tagged glutamate carrier is actively expressed and that neither the His tag nor the Arg2Gly mutation inactivates the transporter.

Glutamate uptake in vesicles derived from DH5α expressing pGltT*nco*I gave similar results, but the initial rate of uptake was significantly lower than observed with GltThis (not shown). This may be caused by a different specific activity of the mutant carriers, different levels of expression, or a different energetic state of the membranes. The latter was clearly the case since uptake of proline catalyzed by the endogenous *E. coli* proline carrier in the two membrane preparations was significantly lower in the membranes expressing GltT*nco*I. Moreover, DH5α cells expressing the latter transporter showed a strongly reduced growth rate, whereas the cells expressing the His-tagged protein showed only a moderately reduced growth rate. The strongly reduced growth rate and the decreased transport activities suggest that overproduction of GltT*nco*I has a negative effect on the cells' metabolism, most likely due to an impaired energy transduction. The His tag may reduce the expression level, resulting in well-coupled membrane vesicles. Since an antibody against GltT is not available to measure the level of expression of GltT*nco*I, a firm conclusion about the effect of the His tag on the specific activity of the glutamate transporter is not possible.

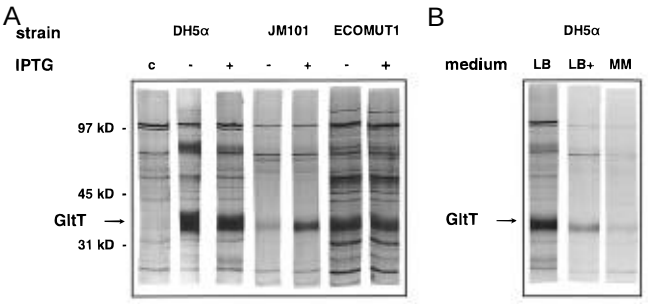

FIGURE 2: Expression levels of GltThis. (A) *E. coli* strains DH5α, JM101, and ECOMUT1 harboring plasmid pGltThis were grown in LB medium in the presence (+) or absence (−) of 0.4 mM IPTG, and the expression level of GltT was determined as described in Materials and Methods. Shown is the silver-stained SDS−PAGE of the protein fraction eluted from a Ni^{2+}-NTA column by 250 mM imidazole. The first lane shows the eluted proteins from DH5α cells harboring the control plasmid pKShis (c). (B) *E. coli* strain DH5α harboring plasmid pGltThis was grown without IPTG in LB medium with (LB+) and without (LB) 1% glucose and in minimal medium containing glycerol (MM).

Plasmid pGltThis was also transformed to *E. coli* ECOMUT1, a strain that lacks the endogenous secondary glutamate carriers (Tolner et al., 1995). Expression of GltThis in ECOMUT1 results in low but significant uptake of glutamate in the membrane vesicles. Apparently, the level of expression in this strain is low (see below).

Optimization of Expression. The level of expression of GltThis in *E. coli* was measured after a rapid partial purification procedure over a Ni^{2+}-NTA affinity resin, followed by SDS−polyacrylamide gel electrophoresis and visualizing the proteins by silver staining (see Materials and Methods). *E. coli* strains DH5α, JM101, and ECOMUT1 were tested for their ability to overexpress GltThis. Expression of GltThis in DH5α cells results in an additional protein band at an apparent molecular mass of 33 kDa that is not observed in the control cells (Figure 2A). An apparent molecular mass of about 33 kDa is typical for an integral membrane protein of about 45 kDa and is in agreement with a similar molecular mass observed after [^{35}S]methionine labeling (Tolner et al., 1992a). A second but much less intense band is visible at about twice the apparent molecular mass and corresponds most likely to the dimeric form of GltT. These results show that the His-tagged GltT molecule binds to the Ni^{2+}-NTA resin. The levels of expression are remarkably different in the different strains. The highest expression is observed in DH5α which is in line with the uptake activity observed in membrane vesicles (Figure 1). Expression in strains JM101 and ECOMUT1 is significantly lower. Induction with IPTG results in higher levels of expression in strain JM101 which overproduces the repressor LacI. In ECOMUT1 and DH5α, the level of expression is not significantly affected in the presence of IPTG. In these strains, the number of repressors relative to the high copy number of the plasmid is likely to be too low to cause a significant inhibition of transcription. The effect of the medium composition on the expression of GltThis in DH5α is analyzed in Figure 2B. The highest expression is observed in the rich LB medium. Addition of glucose represses expression significantly as expected, and hardly any expres-

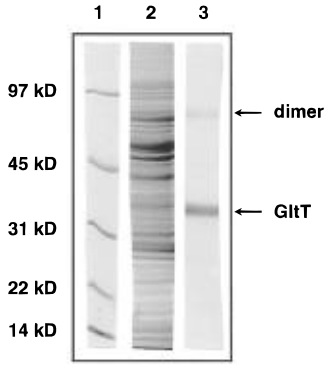

FIGURE 3: SDS−PAGE of purified GltThis. A 12% SDS−PAGE gel was loaded with molecular mass markers (lane 1), 50 μg of French Press-derived membrane vesicles derived from DH5α/pGltThis cells (lane 2, and 3 μg of purified GltThis (lane 3). The gel was stained with Coomassie Brilliant Blue.

Table 3: Effect of Additions during Solubilization, Purification, and Reconstitution on the Yield and Activity of GltThis

addition[a]	yield (μg/mg of membrane protein)	initial uptake rate[b] (pmol μg^{-1} min^{-1})
none	8.5	55.9
glycerol	6.5	109.7
glutamate	6.7	92.6
lipids	7.0	71.8
all	7.2	84.5

[a] Concentrations during solubilization/purification were as follows: glycerol, 10%; lipids, 0.2 mg/mL; and glutamate, 1 mM. Concentrations during reconstitution were as follows: glycerol, 2%; and glutamate, 0.2 mM. [b] The uptake of glutamate was driven by an artificially imposed proton motive and ion motive force as described in the legend to Figure 5.

sion could be observed after growth on a minimal medium with glycerol as the sole carbon and energy source. Similar results were obtained with the other strains (not shown). Strain DH5α harboring plasmid pGltThis grown in LB medium without IPTG was selected for purification of GltThis.

Purification of GltThis. Membranes isolated by French Press treatment from *E. coli* DH5α expressing GltThis were solubilized by the detergent Triton X100 in 50 mM potassium phosphate (pH 8) containing 400 mM NaCl and 20% glycerol. The unsolubilized material was removed by centrifugation. The solubilized membranes were mixed with Ni^{2+}-NTA resin and incubated for 1 h in the cold room under continuous shaking. Subsequently, the mixture was poured into a column and washed with column buffer containing 40 mM imidazole. A small amount of GltThis eluted from the column in the wash step. The bulk of GltThis was eluted with buffer containing 150 mM imidazole. SDS−PAGE of the eluted fractions showed that the protein was essentially pure as judged from both Coomassie Brilliant Blue (Figure 3, lane 3) and silver staining. The amount of GltThis in the membranes is not enough to see the protein after electrophoresis of the membrane fraction (Figure 3, lane 2). The yield of the procedure is typically 30 μg of pure protein from 4 mg of membrane protein which amounts to a fraction of about 0.7% of total membrane protein (Table 3).

An amount of 10 μg of purified GltThis was incubated overnight at 23 °C with 0.6 unit of enterokinase in 20 mM Tris (pH 7.4), 50 mM NaCl, and 0.1% Triton X100 in a total volume of 250 μL and loaded onto a Ni^{2+}-NTA column. Essentially all the GltThis protein bound to the column and

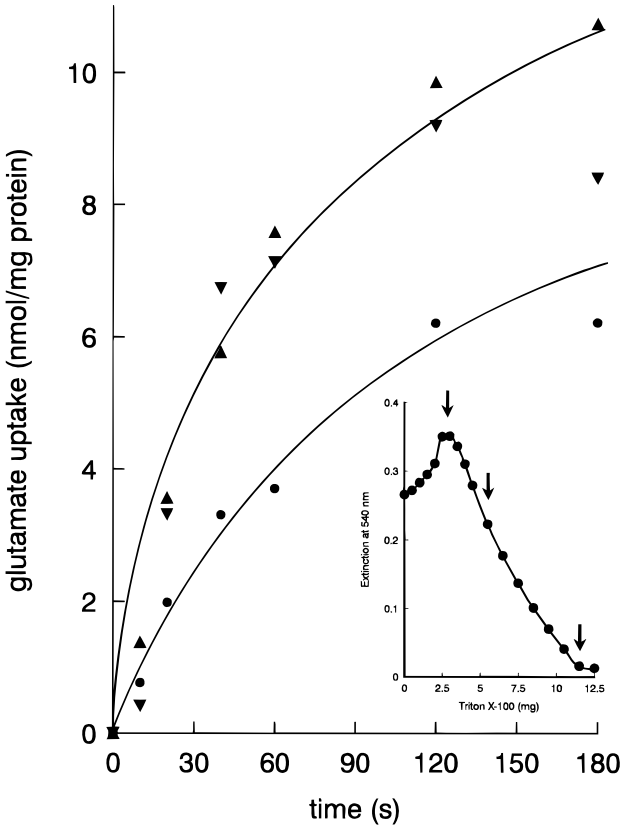

FIGURE 4: Reconstitution of GltT in proteoliposomes. Glutamate counterflow activity was measured in proteoliposomes prepared by mixing purified GltT with liposomes that were saturated with detergent (●), partially solubilized (▲), and completely solubilized (▼). The three states of solubilization are indicated by the arrows from left to right in the inset. (Inset) Light scattering was measured in a suspension of proteoliposomes containing 20 mg of lipid in a total volume of 2.4 mL in the presence of different amounts of Triton X100.

eluted with 150 mM imidazole, indicating that the His tag was not removed by the proteolytic enzyme. Apparently, the enterokinase cleavage site is not accessible to enterokinase in the GltThis fusion protein.

Reconstitution in Proteoliposomes. GltT was reconstituted by mixing the purified protein with preformed liposomes prepared from *E. coli* phospholipids at different stages of solubilization with Triton X100. The solubilization by the detergent was followed by measuring light scattering in the sample spectrophotometrically (Rigaud & Pitard, 1995). Titration of the liposomes with increasing concentrations of Triton X100 results at first in an increase of light scattering caused by the incorporation of detergent molecules in the liposomal bilayer until the liposomes are saturated with detergent (Figure 4, inset). Increasing the concentration further gradually disintegrates the liposomes into mixed detergent/lipid micelles with a concomitant lowering of the light scattering until all liposomes are solubilized. An aliquot of freshly prepared purified GltThis was added at three different points in the solubilization curve: (i) at the point of full saturation, (ii) halfway the breakdown of the liposomes, and (iii) at the point where all liposomes are solubilized (see the arrows in Figure 4). After incubation, the detergent was removed by polystyrene beads and the proteoliposomes were recovered by centrifugation. Figure 4 shows glutamate counterflow activity in the three preparations. Clearly, the reconstituted purified GltThis protein is

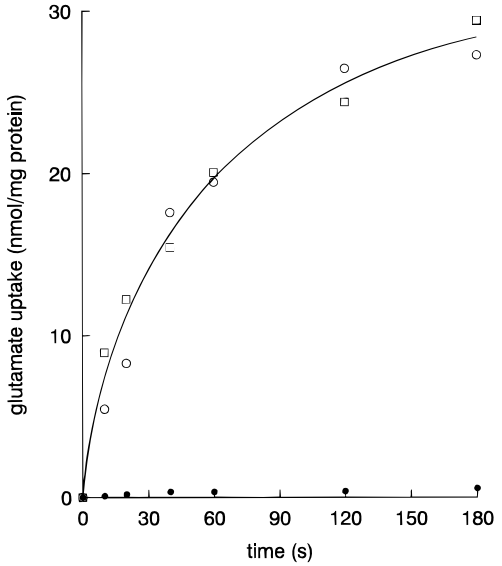

FIGURE 5: Na^+ dependency of reconstituted GltT in proteoliposomes. Glutamate uptake was driven by an electrochemical proton gradient in the absence of Na^+ ions (○) and by both a proton and sodium ion electrochemical gradient (□).

active, and the activity is, considering the complexity of the procedure, most likely not significantly different after the different reconstitution procedures.

Purification and reconstitution of other secondary transporters have been reported to require special precautions to preserve the activity of the protein (see Discussion). These include the presence of glycerol, phospholipids, and substrates or combinations thereof during some or all steps of the solubilization, purification, and reconstitution procedure. The procedure for GltThis purification and reconstitution was repeated in the presence of the additions indicated in Table 3, and the resulting proteoliposomes were assayed for activity. The additions did not affect the purity of the preparation (not shown), and the differences in the yields were within experimental error. The specific initial uptake rates catalyzed by the reconstituted transporters differed maximally by a factor of 2 with no additions and the presence of glycerol giving the lowest and highest activity, respectively. On the other hand, the presence of all additions resulted in an average specific activity, suggesting that the observed differences are not significant.

Cation Specificity. Proteoliposomes of *E. coli* phospholipids containing purified GltT were prepared in the absence of Na^+ ions. The proteoliposomes were loaded with 100 mM potassium acetate and diluted to 100 mM methylglucamine in the presence of valinomycin. Passive diffusion of protonated acetic acid and valinomycin-mediated potassium efflux down their concentration gradients result in the formation of a pH gradient, inside alkaline, and a membrane potential, inside negative. The resulting proton motive force drives the uptake of glutamate into the proteoliposomes (Figure 5, ○), showing that GltT does not require Na^+ to translocate glutamate across the membrane. Dilution of the same proteoliposomes into 100 mM NaCl instead of methylglucamine results, in addition to the generated proton motive force, in an inward-directed Na^+ ion gradient. The additional sodium ion motive force has no effect on the initial rate of glutamate uptake or on the level of accumulation (Figure 5, □). This data indicate that purified GltT reconstituted in *E. coli* phospholipid does not translocate Na^+ ions which is consistent with the cation specificity of GltT in membrane vesicles derived from *E. coli* cells expressing GltT (Tolner et al., 1995).

DISCUSSION

The purification of membrane proteins requires that the proteins are taken out of their natural environment. At least for a while, the phospholipid bilayer is replaced with the unnatural environment of the detergent micelle which may destabilize the protein and result in unfolding. In many purification and reconstitution procedures of transport proteins, special precautions have been described that were essential to keep the protein in the active state [see Poolman and Konings (1993)]. For example, the lactose transporter of *E. coli*, LacY, could only be purified in a functional state when additional phospholipids were added in the detergent-solubilized state (Newman et al., 1981). A high concentration of the osmolyte glycerol was essential in the purification of, for instance, the oxalate transporter of *Oxalobacter formigenes* (Ruan et al., 1992). The presence of substrates of the transporter usually has a stabilizing effect as was claimed for the same carrier and also for the citrate carrier of *Klebsiella pneumoniae (*Pos et al., 1994). In this paper, the purification of the glutamate transporter of the thermophilic bacterium *B. stearothermophilus* is described. Proteins from thermophiles are likely to be more stable at ambient temperatures than proteins from mesophiles, a property that may prove to be of special importance in crystallization studies of membrane proteins. The properties of the GltT protein during the purification and reconstitution procedure support the claim of higher stability in that none of the above precautions seemed to be necessary to keep the protein in the functional state. In this respect, it may be of interest that the alanine carrier of the thermophilic bacterium PS3 also could be purified and reconstituted in the absence of any special additions (Hirata et al., 1984). It is not clear whether the presumed higher stability also relates to the ease by which the protein reconstitutes into liposomes. The stage of solubilization of the preformed liposomes by detergent is much less critical than has been observed for other transport proteins (Rigaud & Pitard, 1995).

Studies in membrane vesicles of *B. stearothermophilus* have demonstrated that GltT catalyzes proton motive force-driven glutamate uptake in the absence of Na^+ ions. In the presence of a Na^+ ion gradient, the uptake activity was significantly higher, especially at elevated temperatures (Tolner et al., 1995). Apparently, GltT translocates glutamate in symport with a proton or a Na^+ ion which places GltT in the same category as, for instance, the melibiose carrier MelB of *E. coli* (Bassilana et al., 1985). In contrast, a glutamate carrier of *E. coli,* GltS, a citrate carrier of *K. pneumoniae,* CitS, and all other amino acid transporters that have been studied in *B. stearothermophilus* translocate their substrates obligatorily coupled to Na^+ ions (Heyne et al., 1991; Tolner et al., 1995; Lolkema et al., 1994a). The differences between these two categories must reflect differences in the structure of the cation binding sites of the proteins. The cation binding pockets of the GltS and CitS type of carriers appear to be more stringent than those of the GltT and MelB types (Lolkema et al., 1994b). The structural difference between binding pockets that accept H^+ or Na^+ may be very small [see for instance Zhang and Fillingame (1995)], and in the case of a pocket that accepts both H^+ and Na^+, a small

change in conformation may result in a significant shift in the relative affinities for the two cations. This may be the explanation for the apparent loss of Na^+ dependency after expression of GltT in *E. coli* when the different lipid environment would cause a change in conformation of the cation binding pocket. The results presented here with the purified protein reconstituted in liposomes prepared from *E. coli* lipid are consistent with the observation made in membrane vesicles derived from *E. coli* cells expressing GltT, i.e. loss of Na^+ ion dependency. At the present, experiments are in progress to reconstitute purified GltT in liposomes prepared from lipids isolated from *B. stearothermophilus*.

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BI953005V